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Selective extraction of phospholipids from soybeans with supercritical carbon dioxide and ethanol.

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Supercritical carbon dioxide (SC-CO_2) is very effective in removing oils from a variety of seed matrices, devoid of any appreciable amount of phospholipids (PLs) content. However, the limited solubility of PLs in SC-CO_2 leaves behind a potentially valuable by-product in the spent seed matrix (defatted soybeans) that could be recovered to economic advantage.

Any PL extraction process from defatted soybeans must maintain the structure and the quality of the PLs and must be compatible with the end use of the seed protein meal as an animal feed or for use in human consumption. For this reason, supercritical fluid extraction is particularly appropriate, since the chosen extracting agent is both environmentally acceptable and non-toxic to food consumers.

Ethanol which is permitted by food safety regulations and which enhances the PL solubility on a thermodynamic basis. PL extraction is possible when ethanol is used as a cosolvent in SC-CO_2.

An initial SC-CO_2 extraction of soybean flakes was performed at 68.2 MPa and 80 °C to extract the oil, leaving the PLs in the defatted soybean flakes (DSF). A second step was performed on the DSF using a X_{eth} = 0.10, varying the pressure from 16.6 to 68.2 MPa and the temperature from 60 to 80 °C. For all SC-CO_2/ethanol extractions, a fraction rich in PLs was obtained. The fractions extracted from defatted soybean flakes (DSF) were dried and then re-dissolved in chloroform before HPLC-ELSD analysis.

Quantitative and qualitative analysis of PLs on soybean seeds, DSF, and different extracted phospholipid fractions were carried out, to ascertain the effort of combinations of extraction pressure and temperature.

Introduction

Phospholipids (PLs) are polar conjugated lipids [1,2]. The terms lecithin and phosphatidylcholine (PC) are often used interchangeably [2]. However, the term lecithin refers to a complex, naturally occurring mixture of PLs, traditionally obtained by water-washing crude vegetable oil and separating and drying the hydrated gums [3]. Therefore, the term lecithin is often used to describe a diverse group of commercially-available PL mixtures, including fractions containing one or more PLs, triglycerides, pigments, carbohydrates, sterols, cerebrosides, in different proportions [3,4].

Previous studies conducted at the National Center for Agricultural Research in Peoria, Illinois, (USA), by Friedrich and co-workers [5,6] showed that supercritical carbon dioxide (SC-CO_2) was very effective in removing oils from a variety of seed matrices, devoid of any appreciable PL content. This property has recently been exploited by List, et al. [7] to continuously degum pre-extracted soybean oil using SC-CO_2. However, the limited solubility of PLs in SC-CO_2 leaves behind a potentially valuable by-product in the spent seed matrix that could be recovered to economic advantage. In addition, any PL recovery process must also be compatible with the end use of the seed protein meal as an animal feed, or for use in human consumption. Since neat CO_2 will not effectively dissolve PL moieties, the

choice of a suitable cosolvent to enhance their solubility must be made not only on a thermodynamic basis, but also with regard to its food safety status , i.e. "Generally Recognized As Safe" (GRAS).

A logical choice for a cosolvent is ethanol, which enjoys GRAS status in the United States. Ethanol has been previously used to fractionate PLs [8], although not in an SFE process; however it has been utilized by Temelli [9] to remove the phospholipids from canola seed using SC-CO_2. Since high pressure phase equilibrium data are available for ethanol/CO_2 mixtures [10], we focused on the use of this cosolvent in fractionating the PL mixtures.

The potential use of SC-CO_2/ethanol mixture for extraction and fractionation of PLs from defatted flaked soybeans seeds has been previously investigated [11, 12]. Initial studies indicated that a small amount of ethanol (5%) in the SC-CO_2 was not enough to extract the PLs, but using a molar fraction of ethanol (X_{eth}) corresponding to 10%, the total recovery of PLs can be increased considerably. Otherwise, the relative amount of phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) in the resulting extract can be varied, using amounts greater than 10%. Since SC-CO_2 extraction allows different kinds of products to be obtained by changing the operating parameters (pressure and temperature), the influence of these processing parameters on the extraction and fractionation was studied.

The objectives of this research were: a) to evaluate the possibility of completely extracting the PLs present in the DSF by using a small amount of ethanol in SC-CO_2 (10 % molar fraction), thereby confirming the possibility of carrying out a dual procedure for the removal of oil and phospholipid fractions from seed matrices using supercritical fluids; b) to fractionate PLs by changing pressure and temperature of SC-CO_2/ethanol mixture. These objectives should be achievable considering that the solubility data for PC and PE in net ethanol (in sub-critical status) show that PC is easier to dissolve than PE.

Experimental procedure

Seeds

The soybean seeds were provided by Mignini S.p.A. (Petrignano d'Assisi, PG, Italy). They had an oil content of 20.6 % by weight, and their moisture content was 12.7 % by weight.

Extraction Procedures

The following two SFE steps were carried out:

Soybean Flakes (SBF)
↓
SC-CO_2 EXTRACTION → Extract (Oil and water mixture)
↓
Defatted Soybean Flakes (DSF)
↓
SC-CO_2/ETHANOL EXTRACTION → Phospholipid-enriched Fraction (PLF)
↓
Exhausted Oil-cake (EO)

The first SC-CO_2 extraction was performed at 32 MPa and 80°C by using a Muller SFE pilot plant (Muller Extract Company GMBH, Coburg, Germany), on 600 g of soybean flakes placed in a 1L nominal vessel. The extract (138.5 g containing 13.7 % moisture) was separated by reducing the pressure at the pressure let down valve to 10 MPa, leaving 445.1 g of DSF in the extractor with a total fat content of 2.0 % by weight and a moisture content of 8.2 % by weight. Table 1 reports the mass balance flow sheet of this first extraction

Table 1 - Mass balance flow sheet (in grams) of CO_2 net extraction.

	IN				OUT			
	WET M.	DRY M.	WATER	OIL	WET M.	DRY M.	WATER	OIL
SBF	600	523.8	76.2	123.6				
EXTRACT					138.5	119.5	19.0	119.5
DSF					445.1	408.6	36.5	8.9
Total	600	523.8	76.2	123.6	583.6	528.1	55.5	128.4
Δ (OUT-IN)					-16.4	+ 4.3	- 20.7	+ 4.8

Subsequent extractions were performed with an Isco SFX™ 3560 extractor equipped with two 100DX syringe pumps (Isco, Inc - Lincoln, NE, U.S.A.). These extractions were performed using an $X_{eth} = 0.10$, varying the pressure from 16.6 to 68.9 MPa, and varying the temperature from 60 to 80° C. The following P-T combinations were utilised:

P (MPa)	68.9	68.9	68.9	52.4	40.7	40.7	40.7	30.1	23.9	23.9	23.9	19.4	16.6	16.6	16.6
T (°C)	80	70	60	80	80	70	60	80	80	70	60	80	80	70	60
$\rho^{(1)}$(g/mL)	0.95	0.97	0.99	0.90	0.85	0.88	0.91	080	0.75	0.80	0.84	0.70	0.65	0.72	0.78

(1): ρ = SC-CO_2/ethanol mixture density

These extractions were conducted for 30 min on samples of 4.5 g of DSF. Each extraction was replicated three times. Both the CO_2 and ethanol syringe pumps were thermostated at 4°C, their flows were chosen to obtain a flow of 2 mL/min for the SC-CO_2/ethanol mixture (X_{eth} = 10%).

PLs were extracted from SBF and DSF by five-fold extraction using the extraction solvent mixture of Bollmann (1:1:1 benzene : ethanol : petroleum ether) [13,14], then dried, and re-dissolved in chloroform.

Reagents

Standard phospholipids, L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC) and L-α-phosphatidic acid sodium salt (PA) from egg yolk, L-α-phosphatidylinositol sodium salt (PI) from soybean, were from Sigma (Sigma-Aldrich, Milano, Italy). All the HPLC-grade and RPE grade solvents were from BDH (Milano, Italy).

Chemical analysis

Dry matter and crude fat were determine on the SBF, Extract, and DSF according to AOCS methods [15,16].

PLs analysis were performed using a Varian 2020 Gradient programmer and two Varian 2010 pumps (Varian, USA) were used. The procedure of Becart et al. [17], slightly modified, was used as follow. A normal phase silica column μPorasil (3.9 cm i.d. × 300 mm) (Waters, Milford, MA, USA) utilizing a Hamilton silica precolumn (Hamilton, Reno, NV, USA) was used. The mobile phase was the following binary gradient:

A): chloroform: methanol: water: 30% ammonium hydroxide at the v/v ratio of 60:34:5.5:0.5, respectively;

B): chloroform: methanol: 30% ammonium hydroxide in the v/v ratio of 80:19.5:0.5, respectively.

The HPLC flow rate was 1 mL/min and the gradient was from 0 to 100% A in 14 min, then 100% A isocratic to 23 min and again to 0% A in 29 min. There was a 5 min isocratic equilibration time before each injection. A 10 μL sample containing 10 to 25 μg of PLs was injected in the column.

Quantitative analyses were carried out for phospholipids where high purity standards were available. Calibration curves for each of the PL standards were run by injected amounts ranging from 1 μg to 10 μg.

An Alltech Varex MKIII ELSD (Alltech Associates Inc., Deerfield, IL, USA) was used to detect the HPLC eluate, nebulized with an air flow of 3.5 mL/min to a drift tube set at 80°C.

The signal output from all the detectors was analyzed using a Varian DMS 654 integration station. Statistical analysis was done using Statgraphics™ software (Statgraphics manual, 1992).

Because this study is part of an international bilateral project, the HPLC analysis was replicated on the extracted PLFs in the laboratories of the National Center for Agricultural Utilization Research, United States Department of Agriculture, Peoria (IL, U.S.A.). There were no consistent discrepancies.

Results

Table 2 reports the amount and the percentage repartitioning of PLs extracted with the Bollmann reagent from SBF and DSF. Table 3 reports the amount and the percentage repartitioning of PLs extracted via SFE at 80°C at different pressures, using a X_{eth} = 10%. All data were normalised to 1 g of DSF. From these experiments, the following considerations can be drawn:

1. Comparing the PLs present in SBF and in DSF, it is possible to see that no PLs were extracted in the first SFE with net CO_2.
2. The highest amount of PLs was extracted at 68.9 MPa, and they are 74.8 % of the total present in the DSF according to the extraction performed using the Bollmann reagent. At this pressure no enrichment of any PL was obtained, i.e. the percentages relative of PE, PC, PI, and PA were the same as obtained using the Bollmann reagent.
3. Decreasing the pressure decreased the amount of PLs extracted, but the relative percentage of each PL changed. In particular, at 19.4 MPa, PC represented 80.1% of the total PLs extracted, while with the Bollmann reagent was only 64.7%.
4. More PI was extracted with the Bollmann reagent than with SC-CO_2/ethanol 10% mixtures. Its maximum extraction yield was obtained at 68.9 MPa (0.26 mg PI/g DSF in comparison with 0.90 mg PI/g DSF extracted with the Bollmann reagent).
5. PA was well extracted from 68.9 MPa to 40.7 MPa (from 0.18 to 0.16 mg PA/g DSF), while only a 0.12 mg PA/g DSF was extracted at 30.1 MPa. Lower pressures do not allowed a significant amount of PA to be extracted.
6. At 16.6 MPa no significant amount of PLs has been extracted.

Table 2 - PLs extracted with the Bollmann reagent from SBF and DSF.

	Phospholipid	mg/ g DSF	%
SBF treated by five fold extraction with Bollmann reagent	PE	2.51	26.6
	PC	6.20	65.6
	PI	0.69	7.2
	PA	0.15	0.6
	Total	**9.54**	**100**
DSF treated by five fold extraction with Bollmann reagent	PE	2.34	24.5
	PC	6.19	64.7
	PI	0.90	9.4
	PA	0.22	1.5
	Total	**9.65**	**100**

Table 3 - PLs extracted from DSF at 80 °C at different pressures, using a X_{eth} = 10%.

PRESSURE	DENSITY	Phospholipid	mg/ g DSF	%
68.9 MPa	0.95 g/mL	PE	1.81	25.1
		PC	4.97	68.8
		PI	0.26	3.6
		PA	0.18	2.5
		Total	**7.22**	**100**
52.4 MPa	0.90 g/mL	PE	1.44	24.0
		PC	4.28	71.4
		PI	0.11	1.8
		PA	0.17	2.8
		Total	**6.00**	**100**
40.7 MPa	0.85 g/mL	PE	0.84	20.8
		PC	2.95	73.4
		PI	0.07	1.8
		PA	0.16	4.0
		Total	**4.02**	**100**
30.1 MPa	0.80 g/mL	PE	0.54	19.0
		PC	2.12	73.9
		PI	0.08	2.9
		PA	0.12	4.2
		Total	**2.87**	**100**
23.9 MPa	0.75 g/mL	PE	0.60	18.5
		PC	2.51	77.7
		PI	0.09	2.8
		PA	0.03	1.0
		Total	**3.23**	**100**
19.4 MPa	0.70 g/mL	PE	0.30	14.5
		PC	1.67	80.1
		PI	0.07	3.4
		PA	0.04	2.0
		Total	**2.09**	**100**
16.6 MPa	0.65 g/mL	PE	0.04	44.1
		PC	0.02	20.7
		PI	0.04	35.2
		PA	0.00	0.0
		Total	**0.10**	**100**

Table 4 reports the amount of PLs extracted from DSF at 68.9, 40.7, and 23.9 MPa at different temperatures (80, 70, and 60 °C). Since no significant amount of PLs was extracted at 16.6 MPa at any temperature, these data are not reported. All data were normalised to 1 g of DSF. From these experiments, the following considerations can be drawn:

Table 4 - PLs extracted from DSF at different pressure and temperatures, using a X_{eth} = 10% .

PRESSURE	T and DENSITY	Phospholipid	mg/g DSF	%
68.9 MPa	80 °C 0.95 (g/mL)	PE	1.81	25.1
		PC	4.97	68.8
		PI	0.26	3.6
		PA	0.18	2.5
		Total	**7.22**	**100**
	70 °C 0.97 (g/mL)	PE	2.15	20.0
		PC	8.20	76.2
		PI	0.19	1.8
		PA	0.23	2.0
		Total	**10.77**	**100**
	60 °C 0.99 (g/mL)	PE	1.80	22.0
		PC	6.22	75.9
		PI	0.14	1.7
		PA	0.03	0.4
		Total	**8.20**	**100**
40.7 MPa	80 °C 0.85 (g/mL)	PE	0.84	20.8
		PC	2.95	73.4
		PI	0.07	1.8
		PA	0.16	4.0
		Total	**4.02**	**100**
	70 °C 0.88 (g/mL)	PE	0.55	17.4
		PC	2.53	80.5
		PI	0.04	1.2
		PA	0.03	0.9
		Total	**3.14**	**100**
	60 °C 0.91 (g/mL)	PE	0.67	19.9
		PC	2.60	77.7
		PI	0.06	1.7
		PA	0.02	0.7
		Total	**3.35**	**100**
23.9 MPa	80 °C 0.75 (g/mL)	PE	0.60	18.5
		PC	2.51	77.7
		PI	0.09	2.8
		PA	0.03	1.0
		Total	**3.22**	**100**
	70 °C 0.80 (g/mL)	PE	0.17	18.1
		PC	0.78	81.9
		PI	0.00	0.0
		PA	0.00	0.0
		Total	**0.95**	**100**
	60 °C 0.84 (g/mL)	PE	0.14	20.5
		PC	0.56	79.5
		PI	0.00	0.0
		PA	0.00	0.0
		Total	**0.70**	**100**

1. At 68.9 MPa, decreasing the temperature increased the amount of extracted PLs. At 70°C more PLs were extracted than when using the Bollmann reagent, i.e. 10.77 mg PLs/g DSF in comparison to 9.65 mg PLs/g DSF (see table 2). At 60°C 8.20 mg PLs/g DSF were extracted. An enrichment of PC has been obtained at 70 and 60 °C.
2. At the lower pressures, reducing the temperature gave an overall reduction of the total extractable PLs.
3. The most interesting enrichment in PC was achieved at 40.7 MPa and 70 °C. In fact at this P and T 3.14 mg PLs/g DSF were extracted, of which 2.53 mg were PC (80.5 % as relative percentage) equal to 40.9 % of the total extractable PC using the Bollmann reagent (6.19 mg PC/g DSF - see table 2). Other phospholipid fractions with more than 80 % PC (as relative percentage) were obtained. They correspond to a lower amount of the total extractable PC:
   - at 19.4 MPa and 80 °C (see table 3) 1.67 mg PC/g DSF (equal to 27.0 % of the total extractable PC using the Bollmann reagent);
   - at 23.9 MPa and 70 °C only 0.78 mg PC/g DSF (equal to 12.6 % of the total extractable PC using the Bollmann reagent).

Conclusions

The above results show the possibility of completely extracting the PLs present in DSF by using a 10 % SC-CO_2/ethanol mixture, thereby confirming the possibility of carrying out a dual procedure for the removal of oil and the phospholipid fractions from soybean seeds using supercritical fluids. This has remarkable interest because the traditional extraction with hexane leaves about 50 % of the total PLs in the spent seed matrix, while the other 50 % are extracted into the hexane. Hence, only this portion of the PLs are recovered from extraction process using hexane to extract the raw oil.

Moreover, by changing the pressure and temperature of a SC-CO_2/ethanol mixture, some PC-enriched PLFs were obtained. These results, and those shown in previous papers where PL extractions were performed by changing the X_{eth} always at 68.2 MPa and 80 °C [11,12], confirm the possibility of obtaining a PC-enriched PLF and yield a fractionated extraction of PLs from DSF.

In the future, research will be initiated to check the influence of the three variables P, T, and X_{eth}. These studies will require some preliminary studies with model systems.

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